Evaluation of the Microlute® CP Reversed Phase (RP) Solid Phase Extraction 96 well-plate by HPLC-MS

Keywords: SPE, reverse phase SPE, solid phase extraction, HPLC-MS, Acidic, Basic, Neutral, Microlute

BACKGROUND

Solid phase extraction (SPE) is a sample preparation method for the clean-up of samples before chromatographic analysis such as HPLC, and GC. SPE offers a number of advantages to the analyst, including less system downtime and troubleshooting, cleaner chromatograms with a reduction of contaminating compounds, and greater reproducible analyte recoveries. Traditionally, SPE methods use loose-filled resins which often create problems such as voids in the sorbent beds leading to channelling and inconsistent flow-through of solutions. This leads to reduced interactions between analytes and the active resin thus leading to inconsistent results and poor analyte recovery.

The Microlute® CP SPE range from Porvair Sciences, offers a unique, solid hybrid polymer structure which is made up of an interconnected network of evenly distributed pores combined with the retentive media (Figure 1). This design enhances the flow-through of samples to maximise interactions between analytes and the solid phase to deliver a highly reproducible SPE method.

This application note demonstrates the robust SPE LC-MS methodology of the Microlute® CP Reverse Phase (RP) SPE 30 mg plates on a range of neutral, acidic, and basic analytes. In addition, comparing recoveries and reproducibility will highlight the performance benefits over loose-filled methods of SPE.

INTRODUCTION

The first SPE sorbent beds were made with bare silica or bonded with functional groups on the surface e.g. C8, C18, phenyl and NH_2. These silica-based resins are associated with the following disadvantages:

1. Instability at extreme pHs (less than pH 2 and greater than pH 7.5).
2. Residual silanol activity causing unwanted secondary interactions.
3. Low retention of polar compounds resulting in low or irreproducible recoveries[1]

Over recent years, significant advantages have been seen from using polymer-based materials. The reasons behind this include polymer morphological features offering high surface area with well-defined porosity. The processes used to synthesise polymer-based sorbents enable incorporation of numerous chemical functionalities into the porous framework (Figure 1). Having the ability to generate such specific and regular functionality gives high retention capacity for different types of compounds (acidic, basic, and neutral compounds) and stability at extreme pHs.[1]

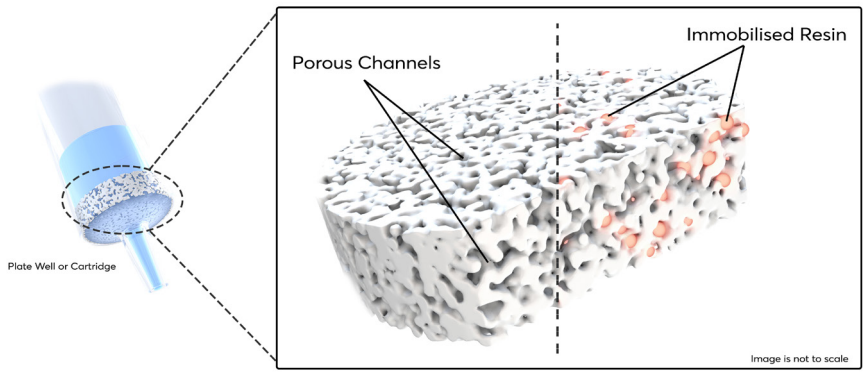

Figure 1. Microlute® Hybrid Technology. A network of porous channels containing immobilised resins for solid phase extraction.

Reversed phase SPE will retain most molecules with hydrophobic properties, allowing for retention of basic, acidic, and neutral compounds. This method is considered the least selective retention mechanism which makes it difficult to differentiate between structurally similar compounds. However, this is also its greatest advantage as it can work with a wide variety of analytes allowing extraction of very structurally diverse compounds in a single SPE run.

Retention of organic analytes from polar solutions onto reversed phase SPE materials occurs due to attractive forces between carbon-hydrogen bonds of the analyte and the functional groups bonded to the sorbent material. These attractive forces known as Van der Waals forces or dispersion forces are interactions that occurs with every compound, at different strengths. The stronger the Van der Waal forces, the more retention is seen on the reversed phase product. The difference in these forces from between compounds allows for clean-up of a sample in either of two ways:

1. Unwanted contaminants are retained on the resin and analytes of interest are eluted.
2. Analytes of interest are retained on the resin, contaminants are washed away and retained analytes are eluted.

Furthermore, polymer-based sorbents avoid problems caused by high active sites which are present in silica-based sorbents. These sites cause unwanted secondary interactions caused by residual silanol groups on the surface to act as acids, leading to unwanted binding of basic analytes or strong hydrogen bonds forming with various compounds. In some cases, this can be useful for separations, but it can vary batch-to-batch or well-to-well. This leads to a potential reduction in both recovery and reproducibility over the course of an SPE run.

Unlike silica-based resins, which can be sensitive to stationary phase collapse if the sorbent bed becomes dry after the condition step, polymer-based sorbents are less susceptible to drying out. This enables quicker SPE method development and gives the analyst ease of mind when performing SPE. In addition, minor packing differences of sorbents into cartridges and wells can cause significant differences in the flow of solutions when performing SPE steps. As a result, analysts using silica-based sorbents will often encounter both problems of flow and drying out of silica and have to resort to eliminating certain wells from their results ultimately leading to inconsistent and unreliable analysis.

Since polymer-based sorbents are better at retaining solutions, all wells and cartridges used can be compared equally irrespective of variable flow-through times thereby, giving the analyst greater confidence in their results.

To demonstrate the sensitivity and robustness of the 30 mg Microlute® CP RP, an SPE experiment was performed using a range of spiked aqueous sample matrices containing neutral, acidic and basic analytes which was then compared against five commercially available 30 mg loose-filled reversed phase products.

EVALUATION OF NEUTRAL ANALYTE RECOVERY AND REPRODUCIBILITY

To demonstrate the sensitivity of the 30 mg polymeric reversed phase Microlute® CP, SPE was carried out on 12 wells using spiked aqueous sample matrices with two neutral analytes. 10 µg of each analyte was loaded onto each well, eluted with organic solvent, dried, and reconstituted before being diluted ready for analysis with LC-MS. The same technique was used to compare performance of equivalent competitor 30 mg polymeric plates.

Experimental

Chemicals:

Hydrocortisone-21-acetate, carbamazepine, caffeine, methanol, ultrapure water.

Sample Preparation:

A stock of 1,000 µg/ml for each group of neutral analytes was made in methanol. A neutral load solution was made by using 500 µL of the stock solution and diluting to 50 ml with water.

Figure 2. Microlute® CP Method for Reversed Phase Solid Phase Extraction for Neutral Analytes.

LC Conditions

LC system	Agilent LC-MS (with a 1260 LC and Single Quadrupole Mass Spectrometer)		
Column	Raptor Biphenyl 30 x 2.1 mm, 1.8 µm		
Column temp.	30°C		
Injection volume	2.00 µL		
Flow rate	400 µL/min		
Mobile phase A	0.1% Formic acid in water		
Mobile phase B	0.1% Formic acid in methanol		
Solvent Composition	Time (min)	A%	B%
	0.00	45	55
	2.50	45	55

Table 1. LC and MS system conditions for chromatographic separation of neutral analytes.

Mass Spectrometer Conditions

Parameter	Value
Gas Temperature	350°C
Gas Flow	13 L/min
Nebulizer	30 psi
Capillary Voltage	4000 V
Dwell Time	150 ms
Fragmentor Voltage	110 V
Scan Type	SIM
Ion Mode	ESI

RESULTS & DISCUSSION

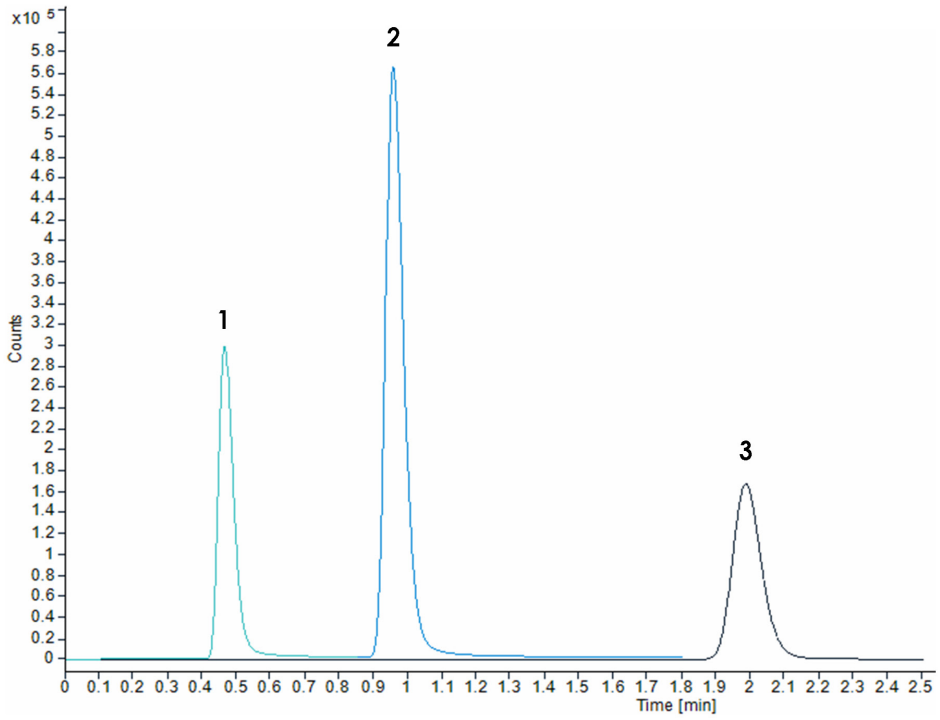

Figure 3. Chromatogram of neutral analytes calibration standard. Peak assignments can be found in Table 2.

Peak Assignment Table

	Compound	Type	R.T (min)	Formula	Molecular Mass	LogP[a]	pKa[a]
1	Caffeine	ISTD	0.48	$C_8H_{10}N_4O_2$	194.19	-0.07	14.0
2	Carbamazepine	Neutral	0.95	$C_{15}H_{12}N_2O$	236.27	2.5	15.6, -3.8*
3	Hydrocortisone-21-acetate	Neutral	2.00	$C_{23}H_{32}O_6$	404.50	2.2	12.61, -2.8*

Table 2. Properties and MS parameters for the neutral compounds analysed - [a] Predicted value from Pubchem[3] *This compound has both acidic and basic groups which are ionisable, pKas are listed for both.

Both carbamazepine and hydrocortisone-21-acetate are neutral compounds with similar hydrophobic properties. Due to this, the recovery of these two compounds depends purely on substantial hydrophobic interactions with the SPE sorbent. Caffeine is a neutral internal standard that was added to the sample after the SPE process.

Recovery Comparison Against Competitors

Recovery is an important metric in any sample preparation method. Higher recoveries allow more sensitive methods along with lower limits of quantification and detection. The data shown in Figure 4 highlights how the Microlute® CP Reversed Phase (RP) product can give very high recoveries for neutral analytes.

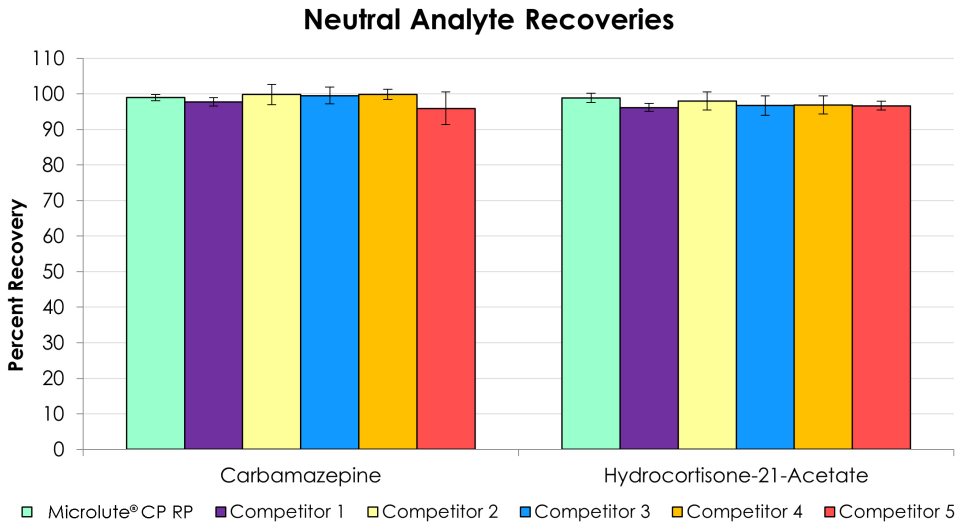

Figure 4. Neutral analyte recovery comparisons against equivalent competitor SPE plates. Number of wells tested = 12. Error bars represent standard deviations.

Figure 4 shows that Microlute® CP RP 30 mg SPE product marginally out-performs all competitors in terms of average recovery of both compounds, with an average recovery value of 98.9%. This is only matched by competitor 2. Where the Microlute® product truly performs better is in how reproducible these recoveries are from well to well.

%RSD Comparison Against Competitors

Reproducibility is needed in a data set to help bring confidence to data that was collected. The Microlute® CP range has been designed with this principle in mind aiding the user to trust the results collected are as precise as possible. Relative standard deviation (%RSD) is used to measure reproducibility in this application note. With %RSD, the lower the value the more reproducible the data set is.

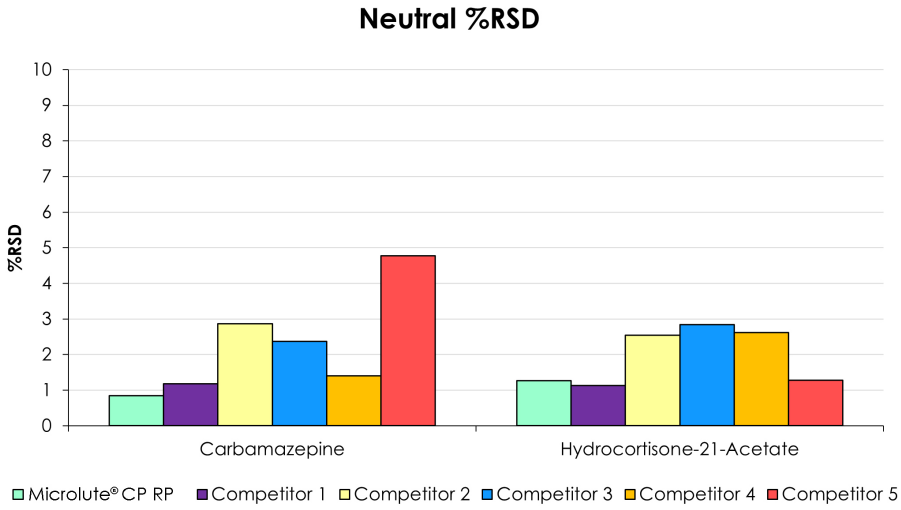

Figure 5. Neutral analyte recovery %RSD comparisons against equivalent competitor SPE products. Number of wells tested = 12.

The neutral analytes %RSD values can be seen in Figure 5. The Microlute® CP RP plate shows significant improvements in reproducibility for carbamazepine when compared to the average of the competitors. This low %RSD of carbamazepine results in the Microlute® CP RP plate having the lowest %RSD on average compared to all competitors and overall best performance when looking at combined average recovery and %RSD of the neutral analytes.

EVALUATION OF ACIDIC ANALYTE RECOVERY AND REPRODUCIBILITY

To demonstrate the sensitivity of the 30 mg polymeric reversed phase Microlute® CP, SPE was carried out on 12 wells using spiked aqueous sample matrices with seven acidic analytes. 10 µg of each analyte was loaded onto each well, eluted with organic solvent, dried, and reconstituted before being diluted ready for analysis with LC-MS. The same technique was used to compare performance of equivalent competitor 30 mg polymeric plates.

Experimental

Chemicals:

Hydrocortisone-21-acetate, 4-pentylbenzoic acid, 4-propylbenzoic acid, diclofenac, ibuprofen, ketoprofen, naproxen, niflumic acid, formic acid, methanol, water, 35% ammonia solution.

Sample Preparation:

A stock of 1000 µg/ml for each of the acidic analytes was made in methanol. An acidic load solution was made by using 500 µL of the stock solution and diluting to 50 ml with water containing 0.1% (v/v) formic acid.

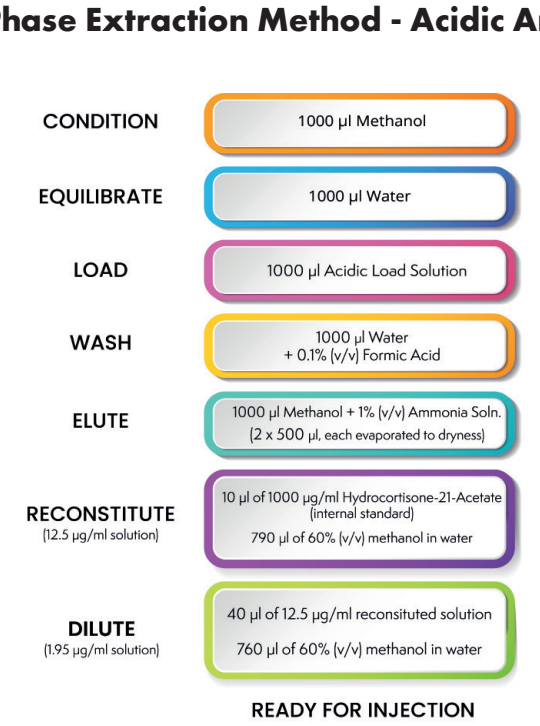

Figure 6. Microlute® CP Method for Reversed Phase Solid Phase Extraction for Acidic Analytes.

LC Conditions

LC system	Agilent LC-MS (with a 1260 LC and Single Quadrupole Mass Spectrometer)		
Column	Raptor Biphenyl 30 x 2.1 mm, 1.8 µm		
Column temperature	30°C		
Injection volume	2.00 µL		
Flow rate	400 µL/min		
Mobile phase A	0.1% Formic acid in water		
Mobile phase B	0.1% Formic acid in methanol		
Solvent Composition	Time (min)	A%	B%
	0.00	35	65
	2.00	35	65

Table 3. LC and MS system conditions for chromatographic separation of acidic analytes.

Mass Spectrometer Conditions

Parameter	Value
Gas Temperature	350°C
Gas Flow	13 L/min
Nebulizer	30 psi
Capillary Voltage	4000 V
Dwell Time	150 ms
Fragmentor Voltage	110 V
Scan Type	SIM
Ion Mode	ESI

*Dwell Time**

**Varied depending on compound detection*

Compound	Dwell Time (ms)
4-Propylbenzoic Acid	75
Niflumic Acid	30
Ketoprofen	30
Naproxen	30
Ibuprofen	30
4-Pentylbenzoic Acid	30
Diclofenac	250

Table 4. Dwell time values for acidic analytes.

RESULTS & DISCUSSION

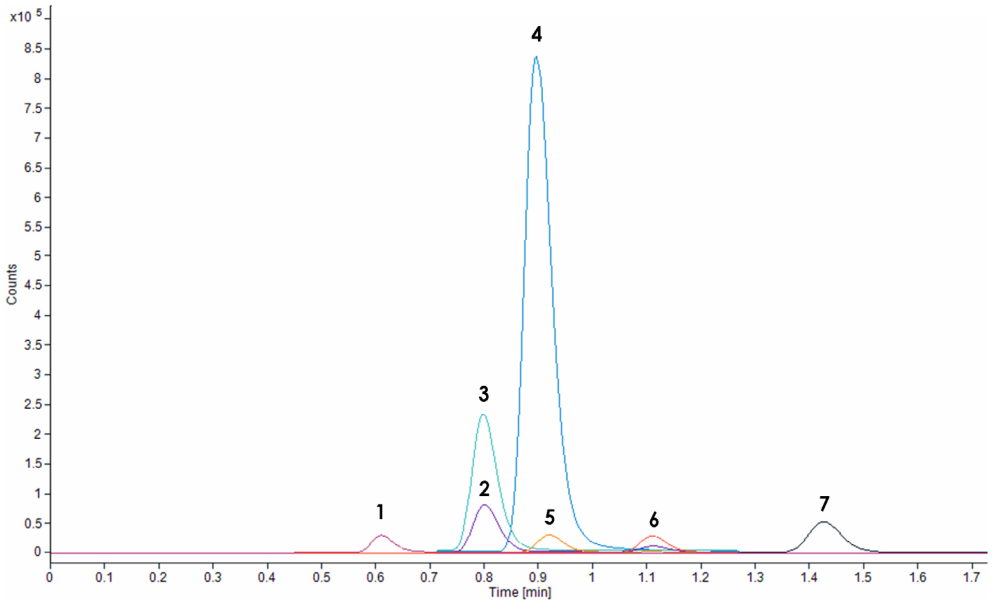

Figure 7. Chromatogram of acidic analytes calibration standard. Peak assignments can be found in Table 5.

Peak Assignment Table

Number	Compound	Type	R.T (min)	Formula	Molecular Mass	LogP[a]	pKa[a]
1	4-Propylbenzoic acid	Acid	0.62	$C_{10}H_{12}O_2$	164.2	3.4	4.4
2	Ketoprofen	Acid	0.80	$C_{16}H_{14}O_3$	254.3	3.1	4.5
3	Naproxen	Acid	0.81	$C_{14}H_{14}O_3$	230.3	3.2	4.2
4	Niflumic acid	Acid	0.90	$C_{13}H_9F_3N_2O_2$	282.2	4.4	1.9, 5.5*
5	Ibuprofen	Acid	0.93	$C_{13}H_{18}O_2$	206.3	4.0	5.3
6	4-Pentylbenzoic acid	Acid	1.12	$C_{12}H_{16}O_2$	192.3	4.5	4.4
7	Diclofenac	Acid	1.42	$C_{14}H_{11}C_{12}NO_2$	296.1	4.5	4.2

*Table 5. Properties and MS parameters for acidic compounds analysed - [a] Predicted value from Pubchem[3] **
This analyte has two ionisable groups present. The pKa of 5.5 is the main ionisable group rendering the compound acidic.

Recovery Comparison Against Competitors

Recovery is an important metric in any sample preparation method. Higher recoveries allow more sensitive methods along with lower limits of quantification and detection. The data shown in Figure 4 highlights how Microlute® CP RP can give very high recoveries for acidic analytes.

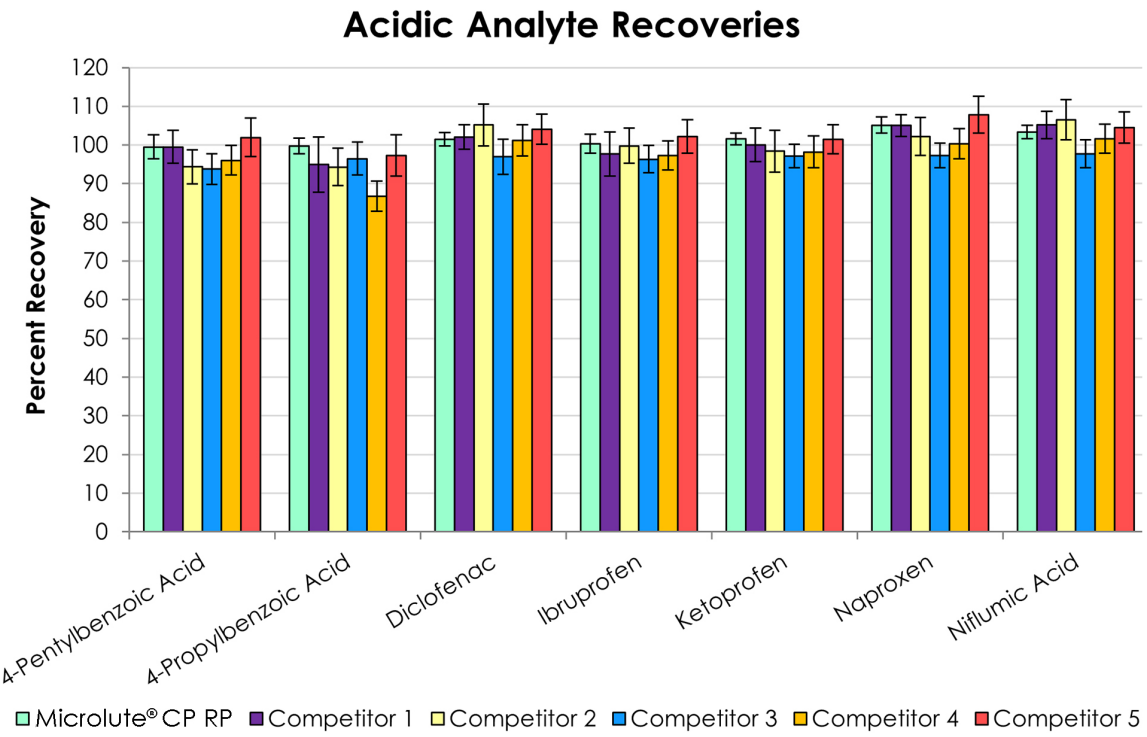

Figure 8. Acidic analyte recovery comparisons against equivalent competitor SPE plates. Number of wells tested = 12. Error bars represent standard deviations.

The acidic analyte recoveries are shown in Figure 8. The Microlute® CP RP plate shows near 100% recoveries for all analytes, showing no significant differences in recoveries compared with competitor 30 mg plates. Some of the results for the competitors did go quite a bit over 100% recovery. This may have been down to error in procedure but is more likely to be due to matrix effects. These can result from extraction on unwanted compounds from the product which end up in the final extract. When these compounds enter the mass spectrometer source they can affect ionisation and lead to differences in peak areas and hence recovery values.

%RSD Comparison Against Competitor Products

Reproducibility is needed in a data set to help bring confidence to data that was collected. The Microlute® CP range has been designed with this principle in mind aiding the user to trust the results collected are as precise as possible. Relative standard deviation (%RSD) is used to measure reproducibility in this application note. With %RSD, the lower the value the more reproducible the data set is.

The acidic analytes %RSD values are shown in Figure 9. The Microlute® plate shows a significant increase in reproducibility for all acidic compounds when compared to every competitor product. The average acidic analyte recovery %RSD value for the Microlute® plate is 2.1%, compared to the most reproducible competitor (competitor 4) with an average of 4.0%.

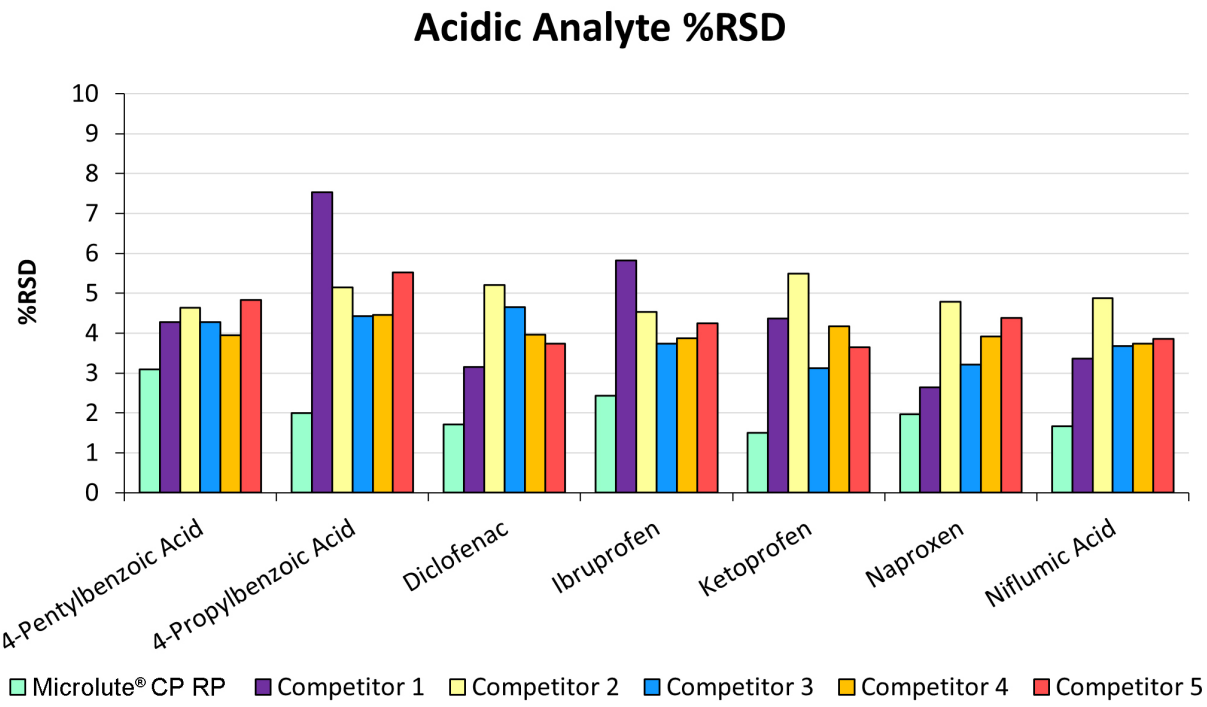

Figure 9. Acidic analyte recovery %RSD comparisons against equivalent competitor SPE products. Number of wells tested = 12.

EVALUATION OF BASIC ANALYTE RECOVERY AND REPRODUCIBILITY

To demonstrate the sensitivity of the 30 mg polymeric reversed phase Microlute® CP, SPE was carried out on 12 wells using spiked aqueous sample matrices with nine basic analytes. 10 µg of each analyte was loaded onto each well, eluted with organic solvent, dried, and reconstituted before being diluted ready for analysis with LC-MS. The same technique was used to compare performance of equivalent competitor 30 mg polymeric plates.

Experimental

Chemicals:

Caffeine, atenolol, salbutamol, propranolol, nortriptyline, protriptyline, imipramine, desipramine, amitriptyline, formic acid, methanol, water, 35% ammonia solution.

Sample Preparation:

A stock of 1,000 µg/ml for each of the basic analytes was made in methanol. A basic load solution was made by using 500 µl stock solution and diluting to 50 ml with water containing 0.1 % (v/v) ammonia solution.

Figure 10. Microlute™ CP Method for Reversed Phase Solid Phase Extraction for Basic Analytes.

LC Conditions

LC system	Agilent LC-MS (with a 1260 LC and Single Quadrupole Mass Spectrometer)		
Column	Raptor Biphenyl 30 x 2.1 mm, 1.8 µm		
Column temperature	45°C		
Injection volume	2.00 µL		
Flow rate	600 µL/min		
Mobile phase A	0.1% Formic acid in water		
Mobile phase B	0.1% Formic acid in methanol		
Solvent Composition	Time (min)	A%	B%
	0.10	95.0	5.0
	4.30	57.5	42.5
	6.50	57.5	42.5
	6.51	20.0	80.0
	8.20	20.0	80.0
	8.21	95.0	5.0
	14.00	95.0	5.0

Table 6. LC and MS system conditions for chromatographic separation of basic analytes.

Mass Spectrometer Conditions

Parameter	Value
Gas Temperature	350°C
Gas Flow	13 L/min
Nebulizer	30 psi
Capillary Voltage	4000 V
Dwell Time	150 ms
Fragmentor Voltage	110 V
Scan Type	SIM
Ion Mode	ESI

*Dwell Time**

**Varied depending on compound detection*

Compound	Dwell Time (ms)
Atenolol	70
Salbutamol	70
Caffeine	150
Propranolol	130
Imipramine	50
Amitriptyline	50
Desipramine	50
Protriptyline	50
Nortriptyline	50

Table 7. Dwell time values for basic analytes.

RESULTS & DISCUSSION

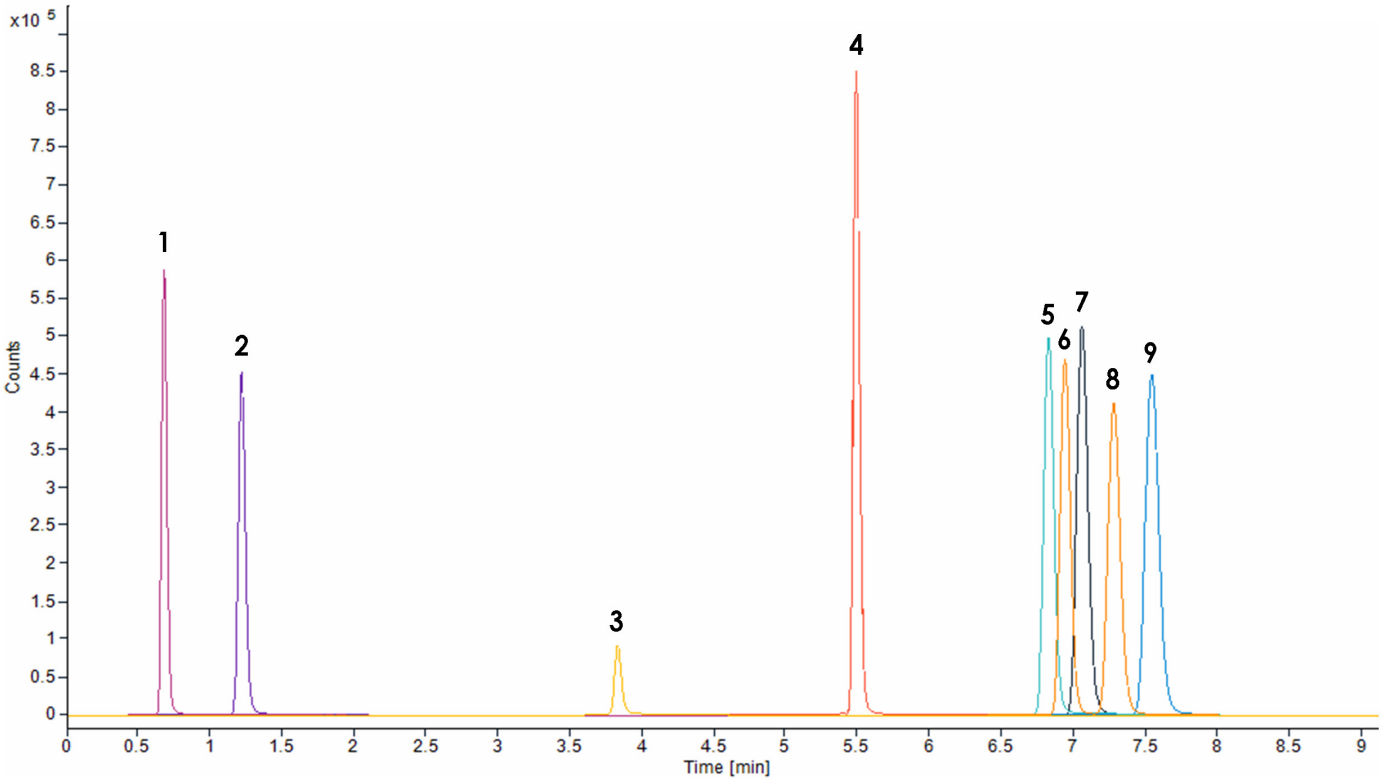

Figure 11. Chromatogram of basic analytes calibration standard. Peak assignments can be found in Table 8.

Peak Assignment Table

Number	Compound	Type	R.T (min)	Formula	Molecular Mass	LogP[a]	pKa[a]
1	Salbutamol	Basic	0.65	$C_{13}H_{21}NO_3$	239.31	0.3	10.3
2	Atenolol	Basic	1.27	$C_{14}H_{22}N_2O_3$	266.34	0.2	10.4
3	Caffeine	ISTD	3.89	$C_8H_{10}N_4O_2$	194.19	-0.1	14.0
4	Propranolol	Basic	5.55	$C_{16}H_{21}NO_2$	259.34	3.0	9.4
5	Desipramine	Basic	6.80	$C_{18}H_{22}N_2$	266.4	4.9	9.6
6	Protriptyline	Basic	7.00	$C_{19}H_{21}N$	263.4	4.4	9.7
7	Imipramine	Basic	7.16	$C_{19}H_{24}N_2$	280.4	4.8	9.4
8	Nortriptyline	Basic	7.41	$C_{19}H_{21}N$	263.4	3.9	10.5
9	Amitriptyline	Basic	7.57	$C_{20}H_{23}N$	277.4	5.0	9.4

Table 8. Properties and MS parameters for basic compounds analysed - [a] Predicted value from Pubchem[3]

Recovery Comparison Against Competitors

Recovery is an important metric in any sample preparation method. Higher recoveries allow more sensitive methods along with lower limits of quantification and detection. The data shown in Figure 12 highlights how the Microlute® CP RP product can give very high recoveries for basic analytes.

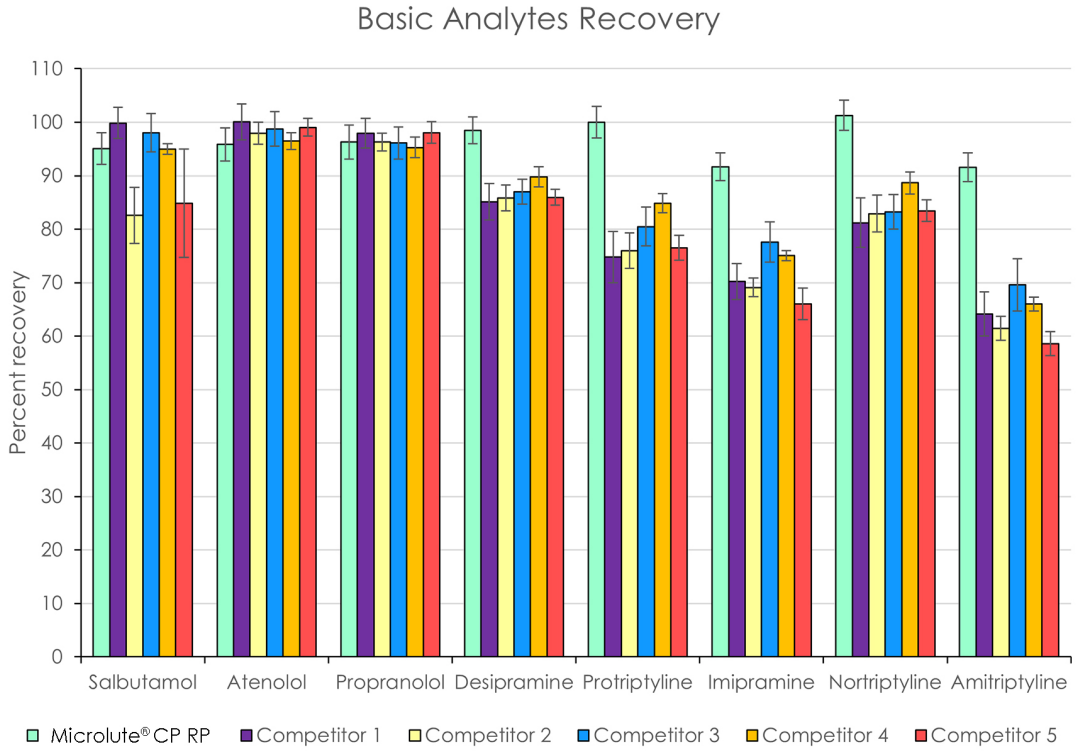

Figure 12. Basic analyte recovery comparisons against equivalent competitor SPE plates. Number of wells tested = 12. Error bars represent standard deviations.

The basic analyte recoveries are shown in Figure 12. Significantly higher recoveries of hydrophobic basic analytes can be seen when compared to competitor plates. For example, the recovery of amitriptyline for the Porvair Microlute® product is 91.6%, comparing this value to the best performing competitor (competitor 3) at 69.6%. This shows an increase in recovery of 31.6%. An increase in recovery of 56.3% can be seen when comparing this recovery against the worst performing competitor.

It might be thought that such an increase in recoveries of more hydrophobic bases would result in a decrease in recoveries for the more hydrophilic bases. From the data collected, this is not seen with there being a great balance in recoveries of hydrophilic bases and hydrophobic bases. The Porvair Microlute® CP RP manages to maintain very high recoveries across the board for the full range of basic compounds.

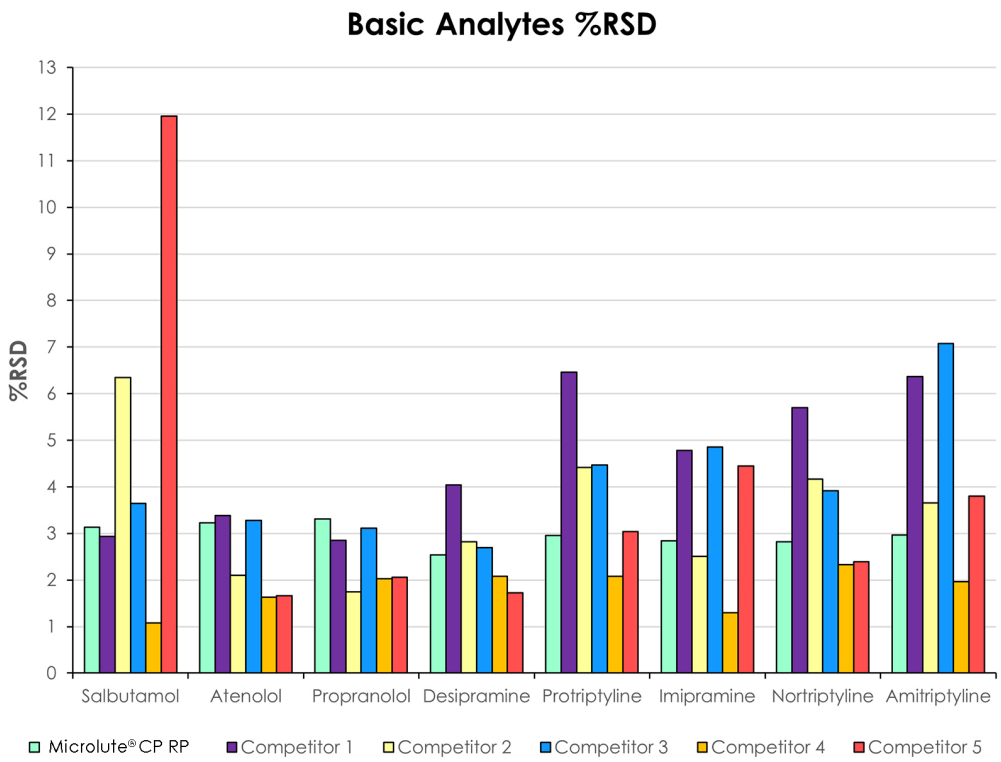

Figure 13. Basic analyte recovery %RSD comparisons against equivalent competitor SPE products. Number of wells tested = 12.Basic

The basic analytes %RSD values can be seen Figure 13. The Porvair Microlute® CP RP obtains %RSD values of less than 3.3% for every compound. This on average beats all competitors except competitor 4. However, when you consolidate both recovery and reproducibility metrics the Porvair Microlute® CP RP combines better recoveries with great reproducibility values to produce the best all-around results for the recovery of basic compounds.

CONCLUSION

The Microlute® CP RP 30 mg 96 well plate can selectively retain and elute a range of neutral, basic and acidic compounds. The %RSD values are on average significantly lower than competitors, giving more reproducible results. The hybrid technology ensures even liquid flow rates throughout the SPE process, which leads to sufficient time for Van der Waals forces of interaction to take place between the sorbent and the analytes. No analyte is lost in the load step of the SPE process leading to high recovery values for all types of compound. The Microlute® CP RP 96 well-plate for solid phase extractions offers significant benefits for the recovery of hydrophobic basic analytes .

References

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3. National Library of Medicine, [Online]. Available: https://pubchem.ncbi.nlm.nih.gov/. [Accessed 2021]

Product Information

Product Number	Description	Format	Pack Qty
PRP030P-001	Microlute® CP RP, 30 mg	96 well plate	1
PPRP0303-050	Microlute® CP RP, 30 mg	3 ml cartridge	50

Find out more about the Microlute® hybrid technology at www.microplates.com/microlute-cp/
Check out the range of Microlute® sample preparation products at www.microplates.com/microlute/

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